

eukaryotic system. Specifically, the Examiner states that that lysostaphin is an anti-microbial protein and that the protein was expressed in active form using mammalian COS-7 tissue cells. Applicant disagrees.

Claim 1 recites a nucleic acid comprising a modified gene encoding a non-mammalian anti-microbial protein, the gene including a sequence that codes for an amino acid sequence that is identical to the anti-microbial protein produced by the natural host except that the coding sequence includes one or more alterations that disrupt one or more mammalian post-translational processing events so that the non-mammalian protein is produced and secreted by mammalian cells in its active form.

The lysostaphin gene described by Williamson et al. was not modified by an alteration that disrupts one or more mammalian post-translational processing events and allows expression and secretion of an active gene product from the cell. As a result, the lysostaphin gene described by Williamson et al. was not secreted in its active form in mammalian cells. For example, William discloses that

"The gene was also expressed transiently from the human cytomegalovirus promoter in COS-7 cells. Active enzyme could be detected in the cell lysate, and the prokaryotic signal appeared to target secretion of active enzyme to the culture medium "  
(Abstract).

However, as it turns out, the lysostaphin protein secreted by the COS-7 cells in these experiments was not active. As described in the specification, "the low level of activity detected by Williamson et al. (*supra*) (less than 1 ng/ml) is likely due either to release from lysed cells of a small amount of protein that escapes glycosylation" (see page 13, lines 4-10). It has now been shown that for active lysostaphin to be secreted in eukaryotic cells, the lysostaphin gene must be

modified to cause one or more disruptions, e.g., amino acid substitutions (see, e.g., page 14, lines 23-24), that prevent mammalian post-translational processing events.

Applicants submit **Exhibit A**, a publication by Kerr et al. that shows that an unaltered lysostaphin protein expressed in mammalian cells lacks staphylolytic activity. Specifically, Kerr et al. teach that

“Transfected cells readily secreted immunoreactive lysostaphin, but western blot analysis indicated that it was ~3kDa larger than the bacterial standard (Fig 1E, line 4). In addition, the protein lacked staphylolytic activity (Fig. 2A). Pretreatment of the sample with N-glycosidase-F followed by western blot analysis revealed a clear reduction in the apparent molecular mass of the expressed protein to that of the lysostaphin standard, indicating that glycosylation was responsible for the aberrant size and inactivity of eukaryotically produced lysostaphin. In an attempt to restore bioactivity, we assembled a new construct designated Sec-Gln<sup>125, 232</sup>-Lys, in which base-pair substitutions were generated to convert Asn125 and Asn232 to glutamine residues. COS-7 cells transfected with Sec-Gln<sup>125, 232</sup>-Lys secreted immunoreactive protein of the appropriate size (Fig. 1E, lane 5), and the medium contained staphylolytic activity (Fig. 2A)” (see page 2, column 1).

The lack of staphylolytic activity described is indicated by the lack of clearing on the lawn of *S. aureus* in Fig. 2A. Disruption of the posttranslational modification sites restores staphylolytic activity. Thus, Kerr et al. demonstrate the necessity of disrupting posttranslational modification events on the lysostaphin protein in order to achieve secretion of the active form of lysostaphin from mammalian cells.

Williamson et al. is completely lacking of any teachings of modifying the lysostaphin gene to obtain secretion an active form of lysostaphin in mammalian cells. There is no teaching in Williamson et al. of disrupting any sites for posttranslational modification to obtain an active form of secreted lysostaphin protein. Indeed, Williamson et al. mistakenly conclude that a

protein secreted in mammalian cells from an unmodified lysostaphin gene is active. Such conclusions are simply incorrect. In light of these facts, Williamson et al. cannot anticipate claims 1-2 and this rejection should be withdrawn.

Claims 1-3 were rejected under 35 U.S.C. 102(e) as being anticipated by Ladner et al. (U.S. Patent No. 6,204,020). The Examiner states that since colony-stimulating factor is an anti-microbial protein and that the protein was expressed in active form using mammalian regulatory sequences, and that glycosylation sites were deleted from the DNA sequence, the disclosure of Ladner et al. is deemed to anticipate the claimed invention.

Claim 1 recites a nucleic acid comprising a modified gene encoding a non-mammalian anti-microbial protein, the gene including a sequence that codes for an amino acid sequence that is identical to the anti-microbial protein produced by the natural host except that the coding sequence includes one or more alterations that disrupt one or more mammalian post-translational processing events so that the non-mammalian protein is produced and secreted by mammalian cells in its active form.

Colony stimulating factor is a *mammalian* protein. The claims are directed to *non-mammalian* proteins. Furthermore, colony-stimulating factor is *not* an anti-microbial protein. Ladner makes this differentiation in teaching that in mammals colony stimulating factor-1 stimulates the growth and development of bone marrow progenitor cells into macrophages and through this action stimulates the immune system, which fights off microbial infection (see column 19, lines 37-42). For example, Ladner notes that administration of colony stimulating factor-1 is a useful adjunct to chemotherapy because chemotherapeutic treatment results in suppression of the immune system (column 19, lines 30-32). Those skilled in the art would

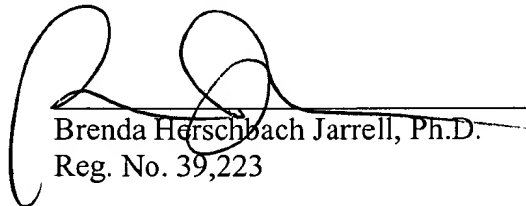
appreciate that the activity of stimulating the immune system is very different from anti-microbial activity. For example, antimicrobial proteins, such as  $\beta$ -lytic protease, lysostaphin,  $\alpha$ -lytic protease, lyt-M, atl, ALE-1, zooA, bacteriocins and others (described in the specification at page 9 line 24 to page 10, line 3) have deleterious effects on the microbial agents themselves; colony stimulating factors do not.

In light of the fact that colony stimulating factor-1 is a mammalian protein and does not have anti-microbial activity, whereas the claims recite non-mammalian anti-microbial proteins, Ladner et al. cannot anticipate claims 1-3. This rejection should be withdrawn.

### Conclusion

Applicant requests entrance of the present Amendment and consideration of the above Remarks. Please charge any fees that may be required, or credit any overpayments, to our Deposit Account No. 03-1721.

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on November 13, 2002  
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# Lysostaphin expression in mammary glands confers protection against staphylococcal infection in transgenic mice

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Infection of the mammary gland, in addition to causing animal distress, is a major economic burden of the dairy industry. *Staphylococcus aureus* is the major contagious mastitis pathogen, accounting for approximately 15–30% of infections, and has proved difficult to control using standard management practices. As a first step toward enhancing mastitis resistance of dairy animals, we report the generation of transgenic mice that secrete a potent anti-staphylococcal protein into milk. The protein, lysostaphin, is a peptidoglycan hydrolase normally produced by *Staphylococcus simulans*. When the native form is secreted by transfected eukaryotic cells it becomes glycosylated and inactive. However, removal of two glycosylation motifs through engineering asparagine to glutamine codon substitutions enables secretion of Gln<sup>125,232</sup>-lysostaphin, a bioactive variant. Three lines of transgenic mice, in which the 5'-flanking region of the ovine  $\beta$ -lactoglobulin gene directed the secretion of Gln<sup>125,232</sup>-lysostaphin into milk, exhibit substantial resistance to an intramammary challenge of 10<sup>4</sup> colony-forming units (c.f.u.) of *S. aureus*, with the highest expressing line being completely resistant. Milk protein content and profiles of transgenic and nontransgenic mice are similar. These results clearly demonstrate the potential of genetic engineering to combat the most prevalent disease of dairy cattle.

Keywords: mastitis, animal biotechnology, antibacterial protein, *Staphylococcus aureus*

Mastitis is an inflammatory reaction of the mammary gland usually caused by a microbial infection, and it is the most prevalent disease of dairy cows<sup>1,2</sup>. This disease, in addition to causing animal distress, is estimated to cost dairy farmers approximately \$200 per cow annually, corresponding to \$1.7 billion annually for the US industry alone. Dairy processors also incur losses from detrimental changes in milk composition that accompany mammary gland inflammation. These changes reduce cheese yield and shelf-life of dairy products<sup>3–6</sup>.

The incidence of contagious mastitis has greatly declined over the last 30 years with implementation of a five-point control plan<sup>7</sup>. However, *S. aureus*, which currently accounts for 15–30% of infections, has proved more difficult to control<sup>8</sup>. The cure rate for treatment of *S. aureus* mastitis with antibiotics is often <15%. This is attributed to incomplete penetration of the antibiotics throughout the gland and the potential survival of bacteria within host cells, leading to a recurrence of disease once treatment has ended<sup>9,10</sup>. Because *S. aureus* mastitis can be induced experimentally with <1,000 organisms<sup>11,12</sup>, a few chronic infections within a herd can provide a persistent bacterial reservoir.

Lysostaphin is a potent peptidoglycan hydrolase secreted by *S. simulans*, having bactericidal activity against *S. aureus* at concentrations of <1  $\mu$ g/ml in milk<sup>11</sup>. The enzyme's staphylococcal activity is mediated by hydrolyzing the polyglycine interpeptide bridges of the cell wall<sup>13</sup>. This specificity restricts its antibacterial activity to *Staphylococcal* sp., having little effect on other mastitis-causing

organisms. However, the enzyme's specificity also makes it an ideal candidate as an antibacterial in milk because it does not seem to degrade milk proteins. The potential of bacterial lysostaphin for therapeutic or prophylactic control of staphylococcal mastitis has been demonstrated, initially in a mouse model<sup>14</sup> and subsequently in dairy cattle<sup>11</sup>.

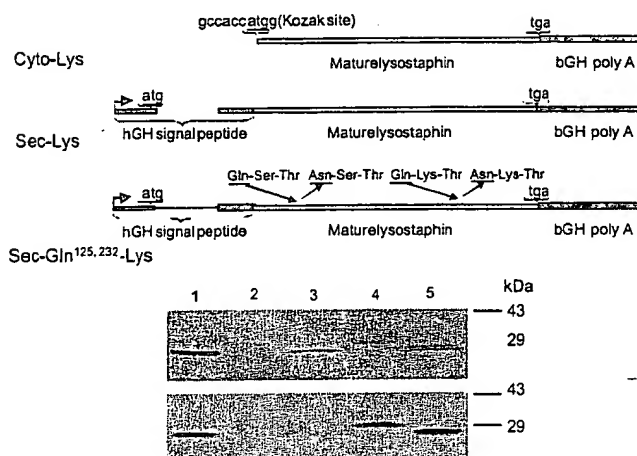
The use of transgenesis to direct expression of a foreign protein into mouse milk was first reported in 1987 by Gordon et al.<sup>15</sup>. Lactation-specific transgenes have since been incorporated into pigs<sup>16</sup>, sheep<sup>17</sup>, goats<sup>18</sup>, and cattle<sup>19</sup>, primarily intended for pharmaceutical interests seeking to generate animal bioreactors. Mammary production of antibacterial proteins for enhanced mastitis resistance has been proposed as a prime agricultural application of this technology<sup>14,20,21</sup>. Antibacterial activity has been observed in milk from transgenic mice that were engineered to produce human lysozyme in milk<sup>22</sup>.

In this report we demonstrate that secretion of a biologically active form of lysostaphin in milk protects transgenic mice against a substantial intramammary challenge of *S. aureus*. These results suggest that a genetic engineering approach will be an effective strategy for enhancing dairy animal welfare and combating the economic burden of mastitis.

## Results

Modification of the bacterial lysostaphin gene for eukaryotic expression. Three lysostaphin-containing constructs were sequen-

## RESEARCH ARTICLES



tially assembled and evaluated following their transfection into COS-7 cells (Fig. 1). Eukaryotic production of cytosolically directed lysostaphin was achieved by addition of a Kozak site to the mature portion of the lysostaphin gene in a construct designated as Cyto-Lys (Fig. 1A). Transfected cells contained appropriately sized, immunoreactive lysostaphin with no apparent secretion of the protein (Fig. 1D, E, lane 3). Staphylytic activity of the cell extract was confirmed with a bacterial plate assay in which the extract was applied to a freshly streaked lawn of *S. aureus* (Fig. 2A). The next construct, designated Sec-Lys, contained the human growth hormone (hGH) signal peptide coding region in place of the Kozak site (Fig. 1B). Transfected cells readily secreted immunoreactive lysostaphin, but western blot analysis indicated that it was ~3 kDa larger than the bacterial standard (Fig. 1E, lane 4). In addition, the protein lacked staphylytic activity (Fig. 2A). Pretreatment of the sample with *N*-glycosidase-F followed by western blot analysis revealed a clear reduction in the apparent molecular mass of the expressed protein to that of the lysostaphin standard, indicating that glycosylation was responsible for the aberrant size and inactivity of eukaryotically produced lysostaphin. In an attempt to restore bioactivity, we assembled a new construct designated Sec-Gln<sup>125,232</sup>-Lys, in which base-pair substitutions were generated to convert Asn125 and Asn232 to glutamine residues. COS-7 cells transfected with Sec-Gln<sup>125,232</sup>-Lys secreted immunoreactive protein of the appropriate size (Fig. 1E, lane 5), and the medium contained staphylytic activity (Fig. 2A).

Generation and characterization of transgenic mice expressing Gln<sup>125,232</sup>-lysostaphin in milk. The Sec-Gln<sup>125,232</sup>-Lys construct was inserted between the 4.2 kilobase 5'-flanking region and the 2.1 kilobase 3'-flanking region of the ovine  $\beta$ -lactoglobulin gene. The entire 7.7 kilo-

base BLG-lysostaphin fusion gene (BLG-Lys) was then used to generate transgenic mice. Eight potential founders carrying the BLG-Lys construct were identified by Southern blot analysis. Two of those animals did not transmit their transgene to progeny. Daughters of three other founders expressed lysostaphin in their milk at very low concentration (<0.020 mg/ml). The other three founders were used to establish lines for further studies. Milk obtained from offspring of one founder contained lysostaphin at  $0.06 \pm 0.01$  mg/ml (mean  $\pm$  s.e.;  $n = 6$ ), and this was designated as the low-expressing line. Representative mice from a second line expressed  $0.13 \pm 0.03$  ( $n = 3$ ) mg/ml in milk, and this was designated the medium-expressing line. Two representative mice from the third line produced 1.3 mg/ml and 0.8 mg/ml in milk and is reported here as the high-expressing line. Northern blot analysis of RNA from various tissues of lactating transgenic mice from each of the lines revealed that lysostaphin expression was almost completely restricted to the mammary gland (data not shown).

Milk from these transgenic mice contains substantial staphylytic activity (Fig. 2B). However, the eukaryotically produced Gln<sup>125,232</sup>-lysostaphin appears to be 5- to 10-fold less active than bacterially derived lysostaphin. The reduced activity was also found in mammary tissue homogenates obtained from lactating transgenic mice. In eight homogenates obtained from four high-line mice the staphylytic activity was  $18 (\pm 3)\%$  of that expected from the concentration of immunoreactive lysostaphin present.

The total protein content of milk samples obtained from the three transgenic lines was quite variable, ranging from 35 to 97 mg/ml ( $n = 11$ ), similar to the range of protein concentrations (35–99 mg/ml) in milk samples from control mice ( $n = 6$ ). The general pattern of milk proteins visualized after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining was similar in all three lines of transgenic mice and in nontransgenic mice (Fig. 3). Western blot analysis of milk from BLG-Lys transgenic mice revealed an appropriately sized lysostaphin band (Fig. 4). Lysostaphin was not detected in milk from nontransgenic mice.

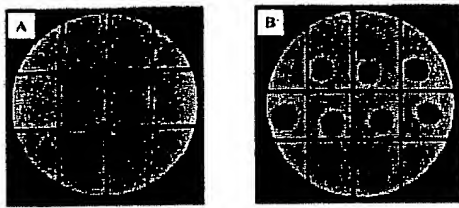
Intramammary challenge of lactating mice with *S. aureus*. Two abdominal mammary glands per mouse of lactating transgenic and nontransgenic mice (day 10 of lactation) were inoculated with *S. aureus* ( $10^4$  c.f.u.). Mice were killed 24 h post infection, and viable *S. aureus* contained in mammary gland homogenates were enumerated. The dose of bacteria was sufficient to cause active intramammary infection in all infused glands of nontransgenic mice (Table 1). In contrast, glands of the high-line BLG-Lys transgenic mice were completely resistant to infection, as were ~40% of glands from the medium and low lines. Furthermore, 17 of 22 control glands were classified as severely infected, based on *S. aureus* recovery. Upon dissec-

**Table 1. Infection status and lysostaphin content of mouse mammary glands after *S. aureus* challenge [AU: OK TO SHORTEN TITLE?]**

Line	Glands infused <sup>a</sup>	Glands infected	Status of infected glands (c.f.u./gland) <sup>b</sup>			Lysostaphin in mammary homogenate ( $\mu$ g/mg protein)
			Severe ( $>10^6$ )	Moderate ( $10^5$ – $10^6$ )	Mild ( $10^2$ – $10^5$ )	
Nontransgenic	22	22	17	4	1	0
BLG-Lys (low)	8	5	0	4	1	$0.27 \pm 0.02$
BLG-Lys (medium)	6	3	0	3	0	$0.86 \pm 0.19$
BLG-Lys (high)	8	0	0	0	0	$12.2 \pm 2.7$

<sup>a</sup>Glands were infused on day 10 of lactation with *S. aureus* ( $10^4$  c.f.u./50  $\mu$ l/gland).

<sup>b</sup>Infection status is based on number of *S. aureus* (c.f.u.) recovered from each gland 24 h post infusion.



**Figure 2.** Bacterial plate assay for staphylolytic activity. Lytic zones developed on a lawn of *S. aureus* 24 h after sample application. (A) Samples derived from transfected COS-7 cells. Top row (left to right), bacterially derived lysostaphin (10, 33, 100, or 333 ng/ml). Middle row (left to right), conditioned medium from COS-7 cells transfected with Cyto-Lys, Sec-Lys, Sec-Gln<sup>125,232</sup>-Lys, or hGH. Bottom row (left to right), cell extracts corresponding to the medium samples in the middle row. (B) Staphylolytic activity in mouse milk. Top row (left to right), bacterially derived lysostaphin (0, 62.5, 125, 250 ng/ml in PBS). Middle row (left to right), skim milk from a BLG-Lys transgenic mouse (medium-expressing line; 190  $\mu$ g Gln<sup>125,232</sup>-lysostaphin per milliliter of milk) diluted 1:50, 1:100, 1:200, or 1:400 in PBS. Bottom row (left to right), skim milk from a nontransgenic mouse diluted 1:50, 1:100, 1:200, or 1:400 in PBS.

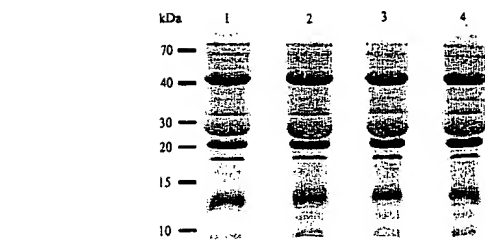
tion these glands had obvious signs of trauma such as a friable texture and a bloody, viscous exudate. In marked contrast, the glands of transgenic mice consistently appeared normal, even in those eight glands from the low and medium lines that contained viable *S. aureus*. Degree of resistance to infection corresponded with the amount of lysostaphin in the glands (Table 1). Within lines, the amount of lysostaphin per milligram of homogenate protein was quite similar. Histological sections further confirmed the infection status (Fig. 5). Severely infected nontransgenic glands were characterized by infiltration of neutrophils and tissue disorganization. In contrast, histological sections of glands from bacterially challenged transgenic mice showed little cellular invasion and a well-preserved alveolar structure.

## Discussion

Infection of the mammary gland is the most prevalent infectious disease of dairy cattle. Mastitis is characterized by an influx of somatic cells, primarily polymorphonuclear neutrophils (PMNs), into milk, and by an increase in milk protease content<sup>23</sup>. Clinical infections are diagnosed by a red, swollen appearance of the gland and by protein flakes or clots in the milk. Milk from clinically infected cows is discarded. Subclinical infections, by definition, show no obvious signs of disease and although milk processing ensures product safety, milk quality is reduced in proportion to the amount of milk protein degradation.

Current therapies for mastitis rely heavily on the use of  $\beta$ -lactam antibiotics such as cepharin and penicillin derivatives. These nonprotein agents have had an enormously beneficial impact on dairy animal health and milk production, although there are drawbacks to their use. In particular, the stability of these compounds has necessitated a strict post-treatment time period during which milk must be discarded to prevent contamination of the milk supply. There is also concern that the use of antibiotics in animal agriculture is contributing to the development of drug-resistant bacteria. This concern is especially relevant for orally active antibiotics that are being used in human medicine. In contrast, protein-based antimicrobials such as lysostaphin will not likely be widely used in human or veterinary medicine because they would be digested and inactivated following oral administration, or run the risk of stimulating an immune response if used intravenously.

The current study has confirmed our hypothesis that transgenic production of lysostaphin by the lactating mammary gland will confer substantial resistance to staphylococcal mastitis<sup>14,21</sup>. Mice from the highest expressing line are completely resistant to experimental

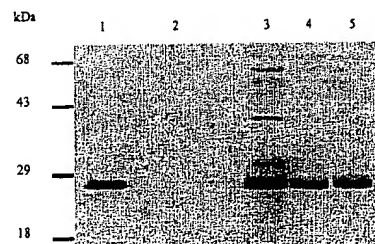


**Figure 3.** SDS-PAGE gel showing protein profiles. Lane 1, molecular weight marker. Lanes 2-4, protein profiles from different samples.

challenge, whereas mice from the medium- and low-expressing lines have substantial resistance. Following the bacterial challenge some glands from low- and medium-expressing transgenic mice did contain viable *S. aureus*. However, none of these glands were visibly altered, whereas most of the glands from nontransgenic mice showed obvious inflammation. The mouse mastitis model employed involves the intramammary infusion of 10,000 c.f.u. of *S. aureus*. This produces an acute infection not likely to occur naturally. With a much lower inoculum it is likely that even the low-expressing line of transgenic mice would have cleared the infection.

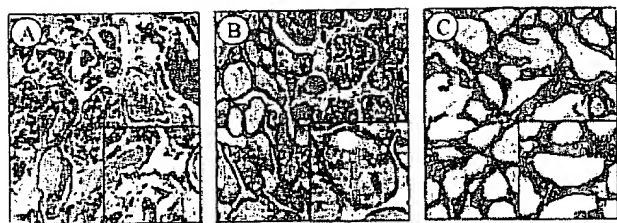
The Gln<sup>125, 232</sup>-lysostaphin variant demonstrates staphylolytic activity at concentrations of <1  $\mu$ g/ml on a lawn of *S. aureus*. This level of foreign protein production by the transgenic mammary gland is easily obtainable. In the present study concentrations of >1 mg/ml were measured. During design of the transgene we chose the proven BLG expression cassette<sup>24</sup> in conjunction with the genomic portion of the hGH gene that encodes the hGH signal peptide. This portion of the hGH gene contributed an intron to the construct that may have enhanced its transgenic production<sup>24,25</sup>. The strategy to replace an asparagine residue with glutamine in the glycosylation sites was based on the similarity of their side groups. This strategy has since been reported as being effective in maintaining the activity of proteins native to prokaryotes when produced by eukaryotic cells<sup>26</sup>. Perhaps other substitution strategies will result in a more active lysostaphin variant.

Production of lysostaphin by the BLG-Lys transgenic mice appears to have little effect on the physiology of the animal, the integrity of the mammary gland, or the milk that it produces. The transgenic animals appear grossly similar to nontransgenic animals, are fertile, and are able to lactate successfully. The mammary glands appeared normal by histological examination, and milk protein profiles were similar to nontransgenic animals. We have not attempted to process the mouse milk into cheese or other fermented products. Lysostaphin activity is very specific for staphylococcal bacteria and



**Figure 4.** SDS-PAGE gel showing protein profiles. Lane 1, molecular weight marker. Lanes 2-5, protein profiles from different samples.

## RESEARCH ARTICLES



**Figure 5.** Hematoxylin and eosin stained sections of lactating mouse mammary tissue obtained 24 h after an intramammary infusion of *S. aureus* ( $10^4$  c.f.u.). (A) Tissue from a severely infected nontransgenic mouse. Marked infiltration of PMNs (dark dots) in alveolar lumen and disorganized alveolar structure are apparent. (B) Tissue from a mildly infected BLG-lys transgenic mouse from the low expression line. Distinct milk fat droplets in lumen and some PMN infiltration are apparent. Structure of alveoli for the most part is intact. (C) Tissue from a noninfected BLG-lys transgenic mouse from the high expression line. Very few nonepithelial cells are visible, and alveolar integrity is normal.

should not have any effect on starter cultures used in the dairy food industry.

Bovine milk normally contains proteins that have antibacterial activity such as lactoferrin, lysozyme, and lactoperoxidase<sup>27,28</sup>, but clearly these are insufficient to prevent mastitis. Efforts to produce antimicrobials and enhance disease resistance have resulted in the generation of transgenic mice and cattle that secrete human lysozyme<sup>22</sup>, human lactoferrin<sup>29</sup>, or bovine tracheal antimicrobial peptide (bTAP) in their milk<sup>30</sup>. Experiments *in vitro* demonstrated that transgenic mouse milk containing human lysozyme (0.38 mg/ml) had slightly enhanced bacteriostatic activity against *S. aureus*, but had no effect on *Escherichia coli*<sup>31</sup>. Challenge studies *in vivo* were not reported. The antimicrobial properties of the lactoferrin-supplemented milks have not been reported. However, the potency of lactoferrin in milk for mastitis-causing organisms is not very strong considering that its concentration in normal milk, which is 200–300 µg/ml, is unable to prevent infection, and its concentration in milk from infected cows often exceeds 1 mg/ml (ref. 32). The antibacterial activity of transgenic mouse milk containing bTAP (5 µg/ml) was not reported, nor were challenge studies done<sup>30</sup>. However, antibacterial activity of bTAP purified from transgenic milk by high-performance liquid chromatography [au: ok?] was detected.

Lactating BLG-Lys transgenic mice have substantial resistance to *S. aureus* infection. However, they will not be protected against non-staphylococcal infection, nor against infection in the nonlactating period, and bacterial development of lysostaphin resistance is a possibility. Solutions to these problems are being pursued. First, antibacterial proteins, with potent activities against other mastitis-causing organisms are known, and some will likely be amenable to a transgenic antimastitis strategy. Second, protection during the nonlactating period could potentially be achieved with a different 5'-regulatory region, possibly one triggered by lactogenesis or involution of the gland. Finally, the possibility of bacterial strains developing resistance to a transgenic strategy may be greatly reduced by the simultaneous production of two or more antibacterial proteins that have different bacteriostatic properties against the same species of pathogen.

### Experimental protocol

**Assembly of lysostaphin constructs.** The coding region of mature lysostaphin was amplified from pCMLEM (ref. 21) and cloned into pcDNA3 (Invitrogen; Carlsbad, CA), creating pCMV-Lys. The expression plasmid pCyto-Lys (cytosolic-lysostaphin) was constructed by inserting a Kozak sequence and a start codon (ATG), 5' to the mature lysostaphin sequence. The plasmid, pSec-Lys (secreted-lysostaphin) was constructed by inserting

the hGH signal-peptide-coding region into pCMV-Lys. The plasmid pSec-Gln<sup>125,232</sup>-Lys encodes two N→Q mutations at positions 125 and 232 of mature lysostaphin. In both cases AAT to CAG codon changes were generated by PCR.

**Generation of BLG-Lys transgenic mice.** The 4.2 kb 5'-flanking region with the first 29 bases of the untranslated region and 2.1 kb of the 3'-flanking region of the ovine BLG gene were obtained from Dr. A.J. Clark (pBJ41; Roslin Institute, UK). A 1.4 kb, *KpnI*-*PvuII* fragment containing the hGH signal peptide region, the modified lysostaphin gene, and the bovine growth hormone (bGH) polyadenylation signal was excised from pSec-Gln<sup>125,232</sup>-Lys and inserted into the *EcoRV* site of pBJ41, which forms the junction between the 5' and 3' components of the BLG gene. The entire 7.7 kb BLG-Gln<sup>125,232</sup>-lysostaphin fusion gene (BLG-Lys) was then used to generate transgenic mice by standard techniques.

**Sample collection.** Conditioned media and cell extracts were obtained 48 h post transfection of COS-7 cells. Cells were transfected in six-well plates by the CaPO<sub>4</sub> technique. Each well was transfected with 31.5 µg of plasmid DNA encoding the various lysostaphin constructs or hGH as a negative control. The cells were maintained in Dulbecco's modified Eagle medium [au: as meant?] with 10% fetal bovine serum without antibiotics. Conditioned medium was removed and cell extracts were prepared by three rounds of freeze-thaw lysis in 0.5 ml PBS. Both were clarified by centrifugation (5 min, 14,000 g) before storage (–20°C [au: as meant?]). The experiments were repeated three times.

Mouse milk samples were obtained at day 10 of lactation<sup>22</sup>. Skim milk was prepared by dilution (1:4 in PBS containing 33 mM EDTA), centrifugation (15 min, 14,000 g, 4°C [au: as meant?]), and then aspiration of milk infranatant for storage (–20°C [au: as meant?])<sup>33</sup>.

**Intramammary challenge with *S. aureus*.** Experimental mastitis was produced by intramammary inoculation essentially as described<sup>14,34</sup>. In the experimental model, two abdominal mammary glands of mice were inoculated with *S. aureus* ( $10^4$  c.f.u./50 µl PBS) on day 10 of lactation. Pups were removed and inoculum was delivered to anesthetized mice by the teat canal with a 100 µl Hamilton syringe fitted with a 34-gauge blunt needle. Sterile India ink (1%), shown in preliminary experiments not to influence *S. aureus* growth, was added to the inoculum as a visual aid to confirm, at necropsy, that infusions were successful. Mice were maintained on an analgesic (buprenorphine) for the 24 h experimental period, then killed. Whole or bisected thoracic glands (L4 and R4) were homogenized in 2 or 1 ml of PBS, respectively. An aliquot was diluted 1:10 in PBS containing 20% glycerol for storage (–20°C [au: as meant?]) until subsequent bacterial enumeration. Viable *S. aureus* in the homogenate were determined by dilution plating on blood agar plates. Colony morphology and hemolytic clearing were used as indicators of *S. aureus*. Histological examination was performed on bisected mammary gland tissue that had been formalin-fixed, paraffin-embedded, sectioned (5 µm), and stained with hematoxylin and eosin.

**Detection of lysostaphin immunoreactivity (western and enzyme-linked immunosorbent assay, ELISA) and bioactivity.** An antibody to purified bacterial lysostaphin (Sigma, St. Louis, MO) was custom developed in a rabbit (R. Sargent Inc., Ramona, CA). The antiserum was then affinity purified by chromatography over lysostaphin-agarose gel, and a portion was then conjugated to horseradish peroxidase (HRP; Bethyl, Montgomery, TX). Western blots of bacterially derived recombinant lysostaphin (10 µl of a 1 µg/ml solution in 0.1% BSA in PBS) were separated on a 12% SDS-PAGE. [AU: Edit ok?] Blots were developed with affinity-purified anti-lysostaphin antibody (1:4,000), then alkaline phosphatase-conjugated anti-rabbit IgG (Sigma), followed by BCIP/NBT [AU: PLEASE SPELL] substrate (Pierce, Rockford, IL). For ELISA, the affinity-purified anti-lysostaphin antibody served as the capture antibody, while the HRP-linked antibody (1:25,000) served for detection. The assay was developed with TMB [AU: PLEASE SPELL] substrate (Pierce), and standards were prepared from recombinant lysostaphin (Sigma). Lysostaphin bioactivity was detected in drops (15 µl) of standards, or samples, that were spotted onto culture plates that had just been streaked with a culture of *S. aureus* (bovine isolate, strain M60). Following an overnight incubation (37°C [au: ok?]), bioactivity was observed as lytic zones in the bacterial lawn. In a second-bioactivity assay the lysis of heat-killed *S. aureus* was followed spectrophotometrically (OD<sub>620</sub>). Recombinant lysostaphin was used to construct a standard curve against which activity in dilutions of mammary homogenates was compared.

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**VERSION WITH MARKINGS SHOWING CHANGES MADE**

**In the Claims:**

Claims 1 and 3 have been amended as follows.

1. (Amended) A nucleic acid comprising a modified gene encoding [an] a non-mammalian anti-microbial protein, [wherein the] the gene including a [coding] sequence that codes for an amino acid sequence that is [from the] identical to the anti-microbial protein produced by the natural host except that the coding sequence includes one or more alterations that disrupt one or more mammalian post-translational processing events so that the non-mammalian protein is produced and secreted by mammalian cells in its active form], but has been modified to allow expression of the active form].
  
3. (Amended) The gene of claim 1 wherein the [modified gene contains mutations that eliminate] alteration eliminates one or more glycosylation sites.